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Specification and Drawings as originally filed with Application for Patent Serial No: **2,219,713**, on [redacted]Y, assignee of Philippe Séguéla and Kazimierz Urbanski, for "DNA Encoding a Human Proton-Gated Ion Channel and Uses Thereof".

Agent certificateur/Certifying Officer

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ABSTRACT OF THE INVENTION

The present invention relates to a novel DNA sequence encoding a novel subtype of human proton-gated channel (ASIC3) ; and uses of the sequence thereof.

DNA ENCODING A HUMAN PROTON-GATED ION CHANNEL AND USES THEREOF

BACKGROUND OF THE INVENTION

(a) Field of the Invention

The invention relates to a DNA sequence encoding a novel subtype of human proton-gated channel; and uses of the sequence thereof.

(b) Description of Prior Art

The neuronal excitation induced by the contact of acid on peripheral nerve endings has been linked to the activation of specific proton-sensitive cation channels expressed in primary sensory neurons of mammals (Rang et al. (1991) *Br. Med. Bull.* **47**:534-548). The prolonged pain associated with the contact of acid on peripheral nerve endings is due to the activation of non-inactivating proton-gated channels. The duration of the acid-induced pain could neither be explained by the properties of the proton-gated channel ASIC1 cloned from rat (Waldmann et al. (1997) *Nature* **386**:173-177) and human (Garcia-Anoveros et al. (1997) *Proc. Ntal. Acad. Sci. (USA)* **94**:1459-1464) central neurons, nor by the properties of the proton-gated channel ASIC2 cloned also from rat (Waldmann et al. (1997) *Nature* **386**:173-177) and human (Price et al. (1996) *J. Biol. Chem.* **271**:7879-7882) central neurons. ASIC1 is sensitive to pH 6.5 and lower but inactivates Waldmann et al. (1997) *Nature* **386**:173-177). ASIC2 is sensitive to pH lower than 6 and inactivates rapidly.

It would be highly desirable to be provided with the primary structure of non-inactivating proton-activated channels from human sensory neurons and means for their functional expression.

SUMMARY OF THE INVENTION

One aim of the present invention is to provide the primary structure and functional expression of a subtype of non-inactivating proton-gated channel from human sensory neurons.

Another aim of the present invention is to provide a DNA sequence encoding a novel subtype of human proton-gated channel.

In accordance with the present invention there is provided an isolated nucleic acid molecule which consists essentially of the nucleotide sequence depicted in Figs. 1A and 1B.

The isolated nucleic acid molecule of the present invention encode a peptide consisting essentially of the amino acid sequence depicted in Figs. 1A and 1B.

In accordance with the present invention there is provided a vector, preferably an expression vector, selected from the group consisting of plasmids, phage, retrovirus, baculovirus and integration elements, which include the isolated nucleic acid molecule of the present invention.

In accordance with the present invention there is provided an isolated nucleic acid molecule, which is capable of hybridizing to the isolated nucleic acid molecule depicted in Figs. 1A and 1B, wherein the hybridization occurs at about 35°C to about 65°C and in 5X SSPC and 50% formamide or equivalent hybridization conditions thereto.

In accordance with the present invention there is provided a method of using the isolated nucleic acid molecule depicted in Figs. 1A and 1B, or a sequence which hybridizes under stringent condition to the sequence depicted in Figs. 1A and 1B, to produce a peptide consisting essentially of the amino acid sequence

depicted in Figs. 1A and 1B, which comprises the steps of:

a) transforming a host with a DNA sequence capable of encoding the peptide;

b) incubating the host under conditions which allows the sequence to be express;

c) isolating the peptide from the host; and

d) recording or imaging the activity of the peptide from the host.

The preferred host is selected from the group consisting of bacteria, yeast, fungi, mammalian cells, plant cells, and insect cells.

In accordance with the present invention there is provided a method of using the peptide encoded by the amino acid sequence depicted in Figs. 1A and 1B or domains of the peptide, to produce antibodies, which comprises the steps of:

a) immunizing a host with the peptide or domains of the peptide for a time sufficient for an immunogenic reaction to occur; and

b) isolating antibodies from the immunized host.

BRIEF DESCRIPTION OF THE DRAWINGS

Figs. 1A and 1B illustrate the primary structure of the cDNA (1732 bases) encoding the full-length human ASIC3 (hASIC3) channel subunit. The coding region of 531 amino acids encoded in the mRNA corresponds to nucleotides 22 to 1614;

Fig. 2 illustrates the recording of non-inactivating cationic current induced by strong acid (pH 4.0) in *Xenopus* oocytes injected with hASIC3 clone alone in pcDNA3 vector; and

Fig. 3 illustrates the recording of non-inactivating cationic current induced by weak acid (pH 6.5)

in *Xenopus* oocytes co-injected with hASIC3 clone and rat P2X2 clone both in pCDNA3 vector.

DETAILED DESCRIPTION OF THE INVENTION

Molecular cloning of hASIC3 and *in vitro* translation

Using the TBLASTN algorithm (Altschul et al. (1990) *J. Mol. Biol.* **215**:403-410), virtual screening of the dbEST database with the conserved domain LXTFPAVTLCNXN of ASIC1 and ASIC2 subunits led to the identification of two human fetal brain EST sequences coding for a novel proton-gated channel subunit (EST IDs # AA449579 and AA429417). The clone tagged by EST #AA449579 was sequenced on both strands and was shown to encode a full-length human proton-gated channel subunit (Figs. 1A and 1B). Characteristic natural and unique restriction sites for ClaI, SmaI, SacI, NcoI, XhoI and XbaI are indicated by arrowheads.

This hASIC3 clone was transferred into the HindIII-NotI sites of eukaryotic vector pcDNA3 (Invitrogen) for CMV-driven heterologous expression in HEK-293 cells and *Xenopus* oocytes. Supercoiled hASIC3 plasmid was used for *in vitro* translation using the TnT system (Promega) with T7 RNA polymerase and [^{35}S]-Cysteine according to manufacturer's specifications. The apparent molecular weight of monomeric hASIC3 subunits was 57±3 kiloDaltons, in excellent agreement with the molecular weight of 58.8 kiloDaltons calculated from the predicted primary sequence of the clone.

Functional expression of hASIC3 in *Xenopus* oocytes

Oocytes surgically removed from mature *Xenopus laevis* frogs were treated 2 hrs at room temperature with type II collagenase (Gibco-BRL) in Barth's solution under agitation. Selected stage IV-V oocytes were defolliculated manually before nuclear microinjection

(Séguéla t al. (1996) *J. Neurosci.* 16:448-455) of 10 ng cDNA of hASIC3 in pcDNA3 vector. After 2-4 days of expression at 19°C in Barth's solution containing 10µg/ml gentamycin, oocytes were recorded in two-electrode voltage-clamp configuration using a OC-725B amplifier (Warner Inst.). Signals were acquired and digitized at 500 Hz using a Macintosh IIci equipped with an A/D card NB-MIO16XL (National Instruments) then traces were post-filtered at 100 Hz in Axograph (Axon Instruments). Acidic solutions titered at room temperature in Ringer's solution containing 115 mM NaCl, 2.5 mM KCl, 1.8 mM $CaCl_2$ in 10 mM HEPES were applied during 10 seconds on oocytes by perfusion in constant flow (10 ml/min). During recording, oocyte membrane was clamped at Vh=-100 mV.

There is shown in Fig. 2 the recording of non-inactivating cationic current induced by strong acid (pH 4.0) in *Xenopus* oocytes injected with hASIC3 clone alone in pcDNA3 vector. These data demonstrate that hASIC3 alone can associate in functional homomeric cation channels.

There is shown in Fig. 3 the recording of non-inactivating cationic current induced by weak acid (pH 6.5) in Xenopus oocytes co-injected with hASIC3 clone and rat P2X2 clone both in pCDNA3 vector. These data demonstrate that the co-expression of hASIC3 and rat P2X2 changes the pH sensitivity of homomeric hASIC3 or leads to the formation of heteromeric pH-sensitive channels.

The present invention will be more readily understood by referring to the following examples which are given to illustrate the invention rather than to limit its scope.

EXAMPLE I

Functional expression of recombinant ASIC3 channels in eukaryotic cells

Development of analgesic therapeutical compounds used for the clinically-relevant pharmacological modulation, inhibition or activation of human ASIC3 channels and homologous receptors.

EXAMPLE II

Uses of antibodies directed against human ASIC3 channel subunits

Polyclonal or monoclonal antibodies can be directed against a bacterial fusion protein containing predicted antigenic domains of hASIC3 subunit, or can be directed against peptides from the predicted amino acid sequence of hASIC3 subunit.

Potential uses:

Regional and cellular in situ immunolocalization of mammalian ASIC3 channels in cells naturally or artificially expressing ASIC3 channels.

Immunoprecipitation of mammalian ASIC3 channels for purification of ASIC3 channels and associated proteins, quantitation of ASIC3 channels and associated proteins.

Western blot detection of mammalian ASIC3 channels from cells naturally or artificially expressing ASIC3 channels.

Identification of members of the mammalian ASIC gene family using antibodies for screening expression cDNA libraries.

EXAMPLE III

Uses of human ASIC3 DNA sequence

Identification of novel members of the mammalian ASIC channel family as potential therapeutic targets using hASIC3 channel subunit sequence for the design of nucleic acid hybridization probe or PCR degenerate oligonucleotide primers. While the invention has been described in connection with specific embodiments thereof, it will be understood that it is capable of further modifications and this application is intended to cover any variations, uses, or adaptations of the invention following, in general, the principles of the invention and including such departures from the present disclosure as come within known or customary practice within the art to which the invention pertains and as may be applied to the essential features hereinbefore set forth, and as follows in the scope of the appended claims.

WE CLAIM:

1. An isolated nucleic acid molecule encoding peptides consisting essentially of the amino acid sequences depicted in Figs. 1A and 1B.

2. The isolated nucleic acid of claim 1, wherein said sequence consists essentially of the nucleotide sequence depicted in Figs. 1A and 1B.

3. The isolated nucleic acid of claim 1 or 2, wherein said sequence further comprises a vector selected from the group consisting of plasmids, phages, virus and integration elements.

4. The isolated nucleic acid of claim 3, wherein said vector is an expression vector.

5. An isolated nucleic acid molecule, which is capable of hybridizing to the isolated nucleic acid molecule of claim 1 or 2, wherein said hybridization occurs at about 35°C to about 65°C and in 5X SSPC and 50% formamide or equivalent hybridization conditions thereto.

6. A method of using the isolated nucleic acid molecule depicted in Figs. 1A and 1B, or a sequence which hybridizes under stringent condition to said sequence depicted in Figs. 1A and 1B, to produce peptides consisting essentially of the amino acid sequences depicted in Figs. 1A and 1B, which comprises the steps of:

a) transforming a host with a DNA sequence capable of encoding said peptide;

b) incubating said host under conditions which allows said sequence to be express;
c) isolating said peptide from said host; and
d) recording or imaging the activity of said peptide from said host.

7. The method of claim 6, wherein said host is selected from the group consisting of bacteria, yeast, fungi, mammalian cells, plant cells, and insect cells.

8. A method of using the peptide encoded by the amino acid sequence depicted in Figs. 1A and 1B or domains of said peptide, to produce antibodies, which comprises the steps of:
a) immunizing a host with said peptide or domains of said peptide for a time sufficient for an immunogenic reaction to occur; and
b) isolating antibodies from said immunized host.

human ASIC3

```
TCGCACGACG CGGTTCTGGC CATGAAGCCC ACCTCAGGCC CAGAGGAGGC CCGGCGGCAG      60
                      M  K  P  T  S  G  P  E  E  A  R  R  Q            13
                      MetLysPro ThrSerGlyP roGluGluAl aArgArgGln
CCCTCGGACA TCCGCGTGTT CGCCAGCAAC TGCTCGATGC ACGGGCTGGG CCACGTCTTC     120
P  S  D  I  R  V  F  A  S  N  C  S  M  H  G  L  G  H  V  F            33
ProSerAspI leArgValPh eAlaSerAsn CysSerMetH isGlyLeuGl yHisValPhe
GGGCCAGGCA GCCTGAGCCT GCGCCGGGGG ATGTGGGCAG CGGCCGTGGT CCTGTCAGTG     180
G  P  G  S  L  S  L  R  R  G  M  W  A  A  A  V  V  L  S  V            53
GlyProGlyS erLeuSerLe uArgArgGly MetTrpAlaA laAlaValVa lLeuSerVal
GCCACCTTCC TCTACCAGGT GGCTGAGAGG GTGCGCTACT ACAGGGAGTT CCACCACCAG     240
A  T  F  L  Y  Q  V  A  E  R  V  R  Y  Y  R  E  F  H  H  Q            73
AlaThrPheL euTyrGlnVa lAlaGluArg ValArgTyrT yrArgGluPh eHisHisGln
ACTGCCCTGG ATGAGCGAGA AAGCCACCGG CTCGTCTTCC CGGCTGTCAC CCTGTGCAAC     300
T  A  L  D  E  R  E  S  H  R  L  V  F  P  A  V  T  L  C  N            93
ThrAlaLeuA spGluArgGl uSerHisArg LeuValPheP roAlaValTh rLeuCysAsn
ATCAACCCAC TGCGCCGCTC GCGCCTAACG CCCAACGACC TGCACTGGGC TGGGTCTGCG     360
I  N  P  L  R  R  S  R  L  T  P  N  D  L  H  W  A  G  S  A           113
IleAsnProL euArgArgSe rArgLeuThr ProAsnAspL euHisTrpAl aGlySerAla
CTGCTGGGCC TGGATCCCGC AGAGCACGCC GCCTTCCTGC GCGCCCTGGG CCGGCCCCCT     420
L  L  G  L  D  P  A  E  H  A  A  F  L  R  A  L  G  R  P  P           133
LeuLeuGlyL euAspProAl aGluHisAla AlaPheLeuA rgAlaLeuGl yArgProPro
GCACCGCCCG GCTTCATGCC CAGTCCCACC TTTGACATGG CGCAACTCTA TGCCCGTGCT     480
A  P  P  G  F  M  P  S  P  T  F  D  M  A  Q  L  Y  A  R  A           153
AlaProProG lyPheMetPr oSerProThr PheAspMetA laGlnLeuTy rAlaArgAla
GGGCACTCCC TGGATGACAT GCTGCTGGAC TGTCGCTTCC GTGGCCAACC TTGTGGGCCT     540
G  H  S  L  D  D  M  L  L  D  C  R  F  R  G  Q  P  C  G  P           173
GlyHisSerL euAspAspMe tLeuLeuAsp CysArgPheA rgGlyGlnPr oCysGlyPro
GAGAACTTCA CCACGATCTT CACCCGGATG GGAAAGTGCT ACACATTTAA CTCTGGCGCT     600
E  N  F  T  T  I  F  T  R  M  G  K  C  Y  T  F  N  S  G  A           193
GluAsnPheT hrThrIlePh eThrArgMet GlyLysCysT yrThrPheAs nSerGlyAla
GATGGGGCAG AGCTGCTCAC CACTACTAGG GGTGGCATGG GCAATGGGCT GGACATCATG     660
D  G  A  E  L  L  T  T  T  R  G  G  M  G  N  G  L  D  I  M           213
AspGlyAlaG luLeuLeuTh rThrThrArg GlyGlyMetG lyAsnGlyLe uAspIleMet
CTGGACGTGC AGCAGGAGGA ATATCTACCT GTGTGGAGGG ACAATGAGGA GACCCCGTTT     720
L  D  V  Q  Q  E  E  Y  L  P  V  W  R  D  N  E  E  T  P  F           233
LeuAspValG lnGlnGluGl uTyrLeuPro ValTrpArgA spAsnGluGl uThrProPhe
                                                    ClaI
GAGGTGGGGA TCCGAGTGCA GATCCACAGC CAGGAGGAGC CGCCCATCAT CGATCAGCTG     780
E  V  G  I  R  V  Q  I  H  S  Q  E  E  P  P  I  I  D  Q  L           253
GluValGlyI leArgValGl nIleHisSer GlnGluGluP roProIleI1 eAspGlnLeu
                  SmaI
GGCTTGGGGG TGTCCCCGGG CTACCAGACC TTTGTTTCTT GCCAGCAGCA GCAGCTGAGC     840
G  L  G  V  S  P  G  Y  Q  T  F  V  S  C  Q  Q  Q  Q  L  S           273
GlyLeuGlyV alSerProGl yTyrGlnThr PheValSerC ysGlnGlnGl nGlnLeuSer
```

Fig. 1A

human ASIC3

```
TTCCTGCCAC CGCCCTGGGG CGATTGCAGT TCAGCATCTC TGAACCCCAA CTATGAGCCA    900
F  L  P  P   P  W  G   D  C  S   S  A  S  L   N  P  N   Y  E  P     293
PheLeuProP roProTrpGl yAspCysSer SerAlaSerL euAsnProAs nTyrGluPro

GAGCCCTCTG ATCCCCTAGG CTCCCCCAGC CCCAGCCCCA GCCCTCCCTA TACCCTTATG    960
E  P  S  D   P  L  G   S  P  S   P  S  P  S   P  P  Y   T  L  M     313
GluProSerA spProLeuGl ySerProSer ProSerProS erProProTy rThrLeuMet

GGGTGTCGCC TGGCCTGCGA AACCCGCTAC GTGGCTCGGA AGTGCGGCTG CCGAATGGTG   1020
G  C  R  L   A  C  E   T  R  Y   V  A  R  K   C  G  C   R  M  V     333
GlyCysArgL euAlaCysGl uThrArgTyr ValAlaArgL ysCysGlyCy sArgMetVal

TACATGCCAG GCGACGTGCC AGTGTGCAGC CCCCAGCAGT ACAAGAACTG TGCCCACCCG   1080
Y  M  P  G   D  V  P   V  C  S   P  Q  Q  Y   K  N  C   A  H  P     353
TyrMetProG lyAspValPr oValCysSer ProGlnGlnT yrLysAsnCy sAlaHisPro

GCCATAGATG CCATCCTTCG CAAGGACTCG TGCGCCTGCC CCAACCCGTG CGCCAGCACG   1140
A  I  D  A   I  L  R   K  D  S   C  A  C  P   N  P  C   A  S  T     373
AlaIleAspA laIleLeuAr gLysAspSer CysAlaCysP roAsnProCy sAlaSerThr
```

NcoI
SacI

```
CGCTACGCCA AGGAGCTCTC CATGGTGCGG ATCCCGAGCC GCGCCGCCGC GCGCTTCCTG   1200
R  Y  A  K   E  L  S   M  V  R   I  P  S  R   A  A  A   R  F  L     393
ArgTyrAlaL ysGluLeuSe rMetValArg IleProSerA rgAlaAlaAl aArgPheLeu

GCCCGGAAGC TCAACCGCAG CGAGGCCTAC ATCGCGGAGA ACGTGCTGGC CCTGGACATC   1260
A  R  K  L   N  R  S   E  A  Y   I  A  E  N   V  L  A   L  D  I     413
AlaArgLysL euAsnArgSe rGluAlaTyr IleAlaGluA snValLeuAl aLeuAspIle

TTCTTTGAGG CCCTCAACTA TGAGACCGTG GAGCAGAAGA AGGCCTATGA GATGTCAGAG   1320
F  P  E  A   L  N  Y   E  T  V   E  Q  K  K   A  Y  E   M  S  E     433
PhePheGluA laLeuAsnTy rGluThrVal GluGlnLysL ysAlaTyrGl uMetSerGlu

CTGCTTGGTG ACATTGGGGG CCAGATGGGC CTTTTCATCG GGGCCAGCCT GCTCACCATC   1380
L  L  G  D   I  G  G   Q  M  G   L  F  I  G   A  S  L   L  T  I     453
LeuLeuGlyA spIleGlyGl yGlnMetGly LeuPheIleG lyAlaSerLe uLeuThrIle
```

XhoI

```
CTCGAGATCC TAGACTACCT CTGTGAGGTG TTCCGAGACA AGGTCCTGGG ATATTTCTGG   1440
L  E  I  L   D  Y  L   C  E  V   P  R  D  K   V  L  G   Y  F  W     473
LeuGluIleL euAspTyrLe uCysGluVal PheArgAspL ysValLeuGl yTyrPheTrp

AACCGACAGC ACTCCCAAAG GCACTCCAGC ACCAATCTGC TTCAGGAAGG GCTGGGCAGC   1500
N  R  Q  H   S  Q  R   H  S  S   T  N  L  L   Q  E  G   L  G  S     493
AsnArgGlnH isSerGlnAr gHisSerSer ThrAsnLeuL euGlnGluGl yLeuGlySer

CATCGAACCC AAGTTCCCCA CCTCAGCCTG GGCCCCAGAC CTCCCACCCC TCCCTGTGCC   1560
H  R  T  Q   V  P  H   L  S  L   G  P  R  P   P  T  P   P  C  A     513
HisArgThrG lnValProHi sLeuSerLeu GlyProArgP roProThrPr oProCysAla
```

XbaI

```
GTCACCAAGA CTCTCTCCGC CTCCCACCGC ACCTGCTACC TTGTCACACA GCTCTAGACC   1620
V  T  K  T   L  S  A   S  H  R   T  C  Y  L   V  T  Q   L  .         531
ValThrLysT hrLeuSerAl aSerHisArg ThrCysTyrL euValThrGl nLeu...

TGCTGTCTGT GTCCTCGGAG CCCCGCCCTG ACATCCTGGA CATGCCTAGC CTGCACGTAG   1680
CTTTTCCGTC TTCACCCCAA ATAAAGTCCT AATGCATCAA AAAAAAAAAA AA            1732
```

Fig. 1B

Non-desensitizing pH-sensitive inward current in Xenopu Oocytes microinjected with hASIC3

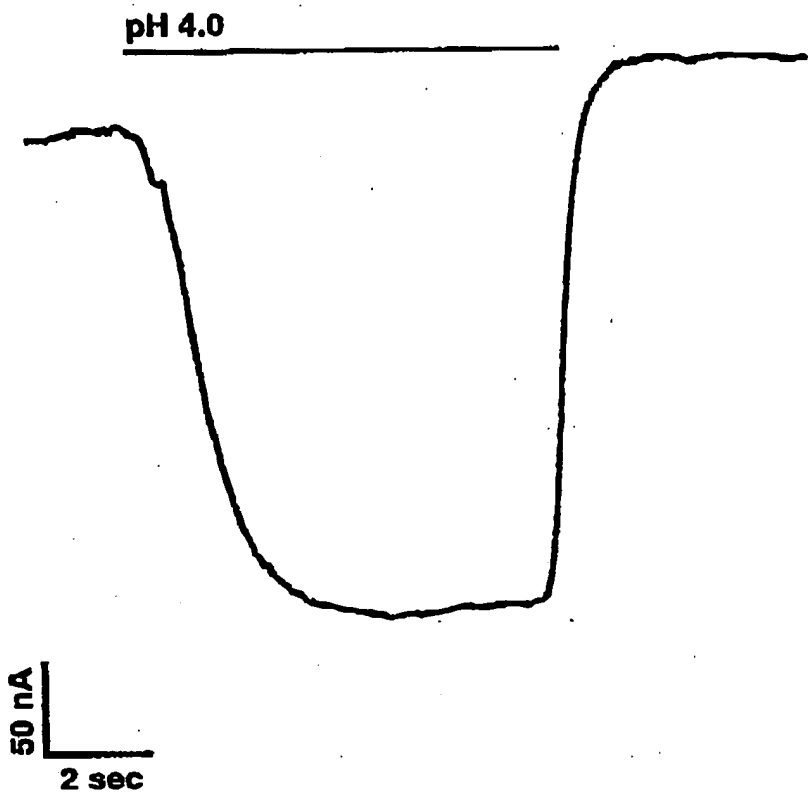

Fig. 2

Non-desensitizing pH-sensitive current in Xenopus oocytes microinjected with human ASIC3 + rat P2X2

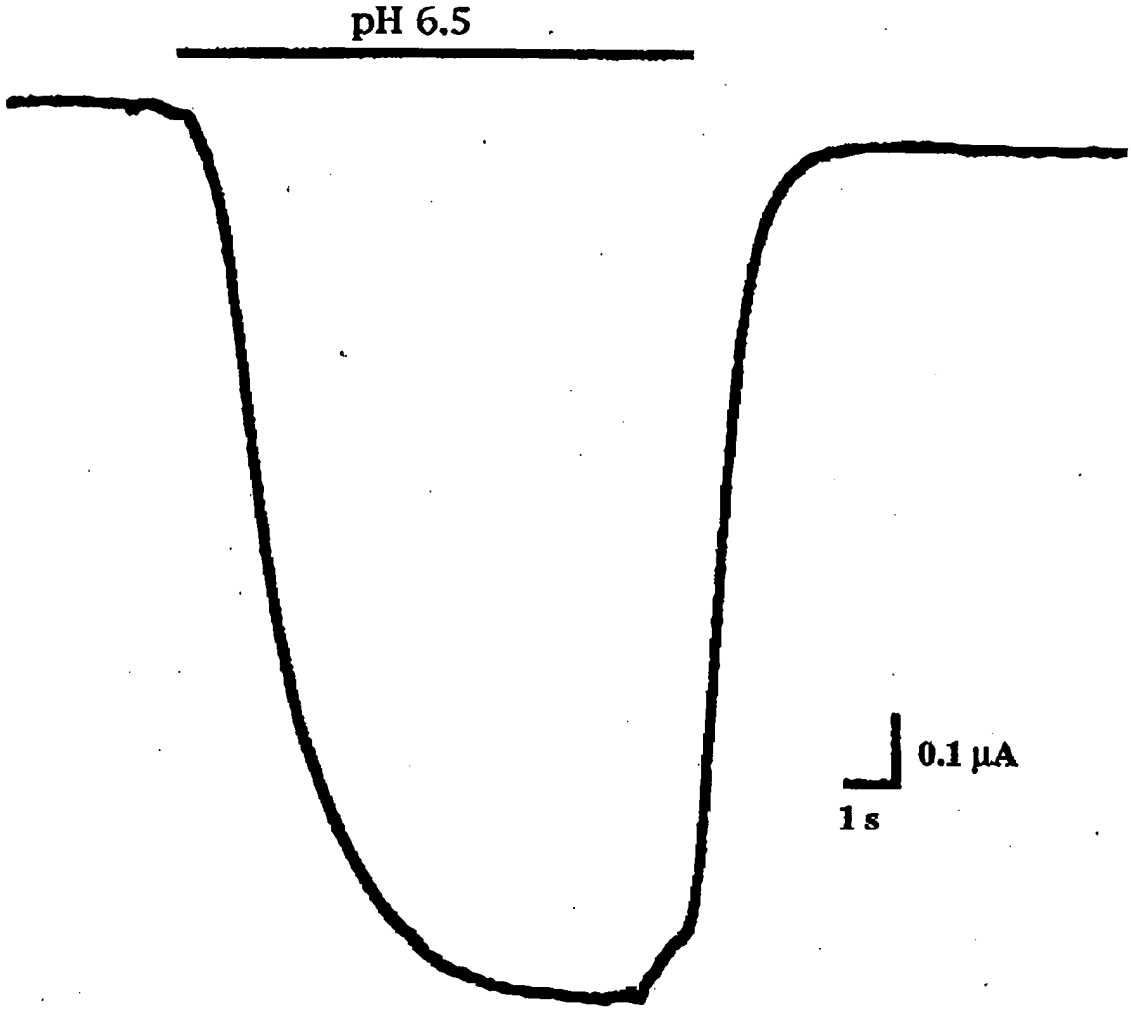

Fig. 3

** TOTAL PAGE.43 **